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RESEARCH ARTICLE

Incorporation of *Moringa oleifera* pods onto breads improves nutrient contents, phytochemicals bioaccessibility and reduces the predicted glycemic index

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ABSTRACT

This work aimed to evaluate *Moringa oleifera* immature pod flour's effect on the nutritional content and bioaccessibility of phytochemicals in partially substituted wheat flour breads. Different bread preparations were made and the most accepted formulation by panelists was chosen for this study. The composition of macro-components, the content of polyphenolic compounds, and the antioxidant capacity of the samples were measured. The bioaccessibility of polyphenolic compounds associated with dietary fiber was also analyzed. Finally, an *in vitro* kinetics on the release of the polyphenolic compounds was conducted. Breads containing 13% of flour from *M. oleifera* immature pods presented the highest acceptance among the substituted breads; meantime, its dietary fiber content was three times higher than white breads. Also, the content of polyphenolic compounds and the antioxidant capacity was higher in the substituted bread, compared to the white ones. Soluble fiber correlated with the increase of polyphenolic compound concentrations in both substituted and white breads. The most significant *in vitro* release of phenolic compounds was shown in those prepared with *M. oleifera*. Taken together, the results demonstrated that breads partially substituted with *M. oleifera* pod flour exhibited the highest nutrient and phytochemical content along with a better bioaccessibility.

Keywords: Dietary fiber; Functional food; Non-conventional food; Wheat-based bread; Polyphenolic compounds

INTRODUCTION

The development of functional food represents a challenge for the food industry and the bioaccessibility of its components should be supported by sufficient scientific evidence. One of the main food macro-components with proven beneficial properties is dietary fiber (DF). DF has a determining role in intestinal health, and its consumption is associated with a lower risk of developing cardiovascular diseases, diabetes, and obesity (Anderson et al., 2009).

Wheat-based breads are one of the main foods worldwide; their consumption dates back to the beginning of society as we know it. However, its consumption volume generates a deficit in DF available amount, compared to other carbohydrates (Juntunen et al., 2003). Several kinds of research have been performed in order to enhance DF content and other components in bread, adding or replacing with matrices such as apple pulp, wheat bran, cane bagasse, unripe banana and pea pod, among others (Massodi, 1998; Sidhu et al., 1999; Sangnark and Noomhorm, 2004; Belghith et al., 2016; Mabogo et al., 2021; Riaz et al., 2022).

On the other hand, the most phytochemicals consumed are the polyphenolic compounds (PPC). PPCs have called society's attention; some studies have associated their consumption with the decreased risk of chronic diseases

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due to their antioxidant characteristics (Pandey and Rizvi, 2009). Some other investigations are currently being conducted providing information on the metabolism and bioaccessibility of PPC, mainly focusing on those PPCs that are extractable by mixtures of water and organic solvents, called soluble or extractable polyphenolics (EPP) (Arranz et al., 2010). PPs that are not traditionally extracted and are subjected to a gradual release either by chewing, acidic pH in the stomach, or the action of digestive enzymes are called non-extractable polyphenolic compounds (NEPP) (Pérez-Jiménez et al., 2013). Recently, it has been reported that NEPPs are directly related to DF and can reach the colon slightly altered after digestion, where they can exert their biological action when fermented by the bacterial microflora (Saura-Calixto, 2011). Nutritionally, these two components can be treated jointly.

There exist vast information on the composition of *M. oleifera* plants and have been characterized by their well-balanced nutritional value, mainly by their high contents of protein, DF and PPC (Sánchez-Machado et al., 2009; Saini et al., 2016).

Previously, several plant tissues and components, mainly from the leaves (foliage) and seeds, have been used as supplements in preparing bakery products. It has been found that the nutritional value of plant-derived bakery products has notably increased and been accepted by consumers (Ongunsina et al., 2011; Sengev et al., 2013; El-Gammal et al., 2016; Mushtaq et al., 2018; Bourekoua et al., 2018). However, the information regarding using pods as additives in the abovementioned products is scarce. In this research, we prepared breads using partially *M. oleifera* immature pod flour, instead of wheat flour as an ingredient. Based on previous described evidence, we considered that the added ingredient would increase the antioxidant compounds and fiber of the developed functional food. Thererfore, the objective was to evaluate the effect of the flour prepared from immature pods of *M. oleifera* and their associated DF on the release and availability of PPCs in breads. Furthermore, the macro-components and PPE were determined and the sensory evaluation of the product was also analyzed.

MATERIALS AND METHODS

Sample preparation

Immature pod samples of *M. oleifera* were collected from domestic crops in the southern region of the State of Sonora, Mexico (36°54'39"N, 109°37'31"O). At the laboratory, they were washed and air-dried, away from the sunlight. The dried samples were then ground and sieved (420 μm mesh) and stored in airtight containers at −20 °C for further analysis.

Formulation of breads and sensory evaluation

An automatic equipment was used to make bread formulations (Hamilton Beach®, Glen Allen, VA, USA) at 150 °C for 3 h. The ingredients used for the formulations were: water (250 mL), salt (5.6 g), sugar (15 g), melted butter (25 g), wheat flour (WF) (400 g), and yeast (4.14 g). For the formulations, a partial substitution of the wheat flour was performed using *M. oleifera* pod flour at 5-15% (MPF). For sensory evaluation, the bread samples were cut into squares (1.5 × 1.5 cm) and placed in disposable cups with lids. A paired preference test with non-acceptance option was performed on untrained panelists (n=140) (Islas-Rubio et al., 2012).

Analysis of macro-components, PPC and antioxidant activity

Moisture (925.09), ash (942.05), lipids (920.39) and total protein (955.04) content were analyzed by the methodologies proposed by AOAC in triplicates (AOAC, 2000). Dietary fiber was analyzed by the AOAC enzymatic-gravimetric method (985.29) (AOAC, 2000).

The total soluble polyphenols (TSPPC), total flavonoid content (TFC) and antioxidant capacity were analyzed followed the procedure propused by Núñez-Gastélum et al. (2015). TSPPC was quantified using a gallic acid calibration curve and expressed in milligrams of gallic acid equivalents per gram of dry sample (mg GAE/g). For the measurement of TFC, catechin was used as the standard and the results were expressed as mg of catechin equivalents per gram of dry sample (DS) (mg EC/g). The antioxidant capacity was analyzed by the ABTS and FRAP methods. Both methods were determined using a standard curve of a standard Trolox solution. Results were expressed as mM Trolox equivalents per g (mM TE/g) for both methods.

Bioaccessibility and kinetics of release of PPC in an *in vitro* model

The bioaccessibility and release kinetics of PPC in bread samples were determined following the procedure proposed by Blancas-Benítez et al. (2015). For bioaccessibility, samples were treated sequentially with pepsin, pancreatin and α-amylase (p7000, P1750, A6255, Sigma, St. Louis MO, USA). PPCs associated with the insoluble indigestible fraction (PPC-IIF), PPC released in the intestinal fraction (PPC-IntF) and PPCs associated with the soluble indigested fraction (PPC-SIF) were determined. The percentage of bioaccessibility was calculated according to:

$$\text{PPC Bioaccessibility}(\%) = \frac{(\text{PPC - Int}) - (\text{PPC - SIF})}{(\text{PPC - Int}) + (\text{PPC - IIF})} \times 100 \quad (1)$$

The gastric stage was simulated using pepsin to perform the release kinetics of the phenolic compounds. First, the intestinal phase was conducted in dialysis tubes with cellulose membranes. Subsequently, α-amylase solution was added. Finally, TSPPC was determined by taking aliquots were taken every 30 min. The release rate during *in vitro* digestion was calculated according to the following formula:

$$Vf = \Sigma\left(\frac{\ddot{A}C}{\ddot{A}t}\right) \quad (2)$$

Where: ΔC is the difference in the content of total polyphenolic compounds between one measurement and another, Δt is the time difference (min) between one measurement and another, and V_f is the final release rate (mg GAE/min).

In vitro method for predicting Glycemic Index (pGI)

The pIG tests were carried out following the method of Granfeldt et al. (1992). Six volunteers participated who attended on fasting conditions and without having brushed their teeth. Volunteers chewed the sample, or control sample (Bimbo® white bread), for 15 s and then the chewed sample was spitted into a pepsin solution (50 mg dissolved in 6 mL of phosphate buffer, pH 6.9). The volunteers rinsed their mouths with 5 mL of phosphate buffer and the liquid was individually collected. The pH was adjusted to 1.5 and the samples were incubated for 30 min at 37 °C with constant shaking. Then, the pH was adjusted to 6.9 and the samples were transferred to the cellulose dialysis membranes previously hydrated in phosphate buffer (pH 6.9). To each sample, 1 mL of pancreatic α-amylase solution (50 µL of pancreatic α-amylase in 7 mL of phosphate buffer, pH 6.9, 110 U sigma) was added in a final volume of 30 mL. Dialysis membranes were suspended in containers containing 800 mL of phosphate buffer (pH 6.9) and incubated at 37 °C and constant shaking for 120 min. The available starch was determined spectrophotometrically taking aliquots (100 µL) in Eppendorf tubes at 0, 30, 60, 120, 150 and 180 min intervals, then were mixed with 1 mL of absolute ethanol; using a glucose standard curve (Holm et al., 1986). The areas under the curve (AUC) of the hydrolysis were obtained using the following equation (Equation 3):

$$AUC = C(t_f - t_0) - \left(\frac{C}{K}\right)\left(1 - e^{-Kt}\right) - K(t_r - t_0) \quad (3)$$

where AUC is the area under the curve, C is the percentage of starch hydrolyzed at time t (min), t_f and t_0 are the equilibrium percentage of starch hydrolyzed after 180 min, respectively; K is the kinetic constant, and tr is the time of reaction.

The hydrolysis index (HI) is the ratio of the area under the hydrolysis curve of the sample (AUC_s) to the area under the hydrolysis curve of white bread as a reference sample (AUC_c) (Equation 4). Finally, the pGI was calculated using the equation established by Goñi et al. (1997); pGI = 39.7 + 0.548HI.

$$HI = \frac{AUCs}{AUC_c} \quad (4)$$

Statistical analysis

For sensory evaluation, a Chi-square test was performed, and data were analyzed whit program XLSTAT version 2016.5 (Addinsoft, Paris, France) at 5% significance level. All other data were compared by Tukey test ($p<0.05$) using program SPSS version 24 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Formulation of breads and sensory evaluation

We considered that a substitution that produces a reduction of more than 50% on the substituted bread height with respect to the wheat bread would be the maimum limit of addition of MPF. A preference test was carried out with the two maximum concentrations 12 and 13%, since the purpose was to know the greatest amount of substituent that we could add without affecting the preference of consumers (Additional information). Although it was not significant, the BMPF 13% was used as we hypothesized that at higher moringa flour substitutions, greater effects could be observed. On average, the white bread (WB) height was 16 cm, in contrast to the 8 cm of the bread added with MPF (BMPF). Sánchez et al. (2008) have referred that it is difficult to get comparable dimensions in partial or complete substituted-wheat flour bread compared to the white counterparts, using identical amounts of ingredients. The latter mainly because other flours cannot form a continuous phase, preventing leavening.

Analysis of macro-components of flours and breads

Proximal analysis of the flours and the breads used in this research was performed to determine their chemical composition (Table 1). Regarding flours, a significant difference was found in the content of moisture, protein, total ash, fiber, and total carbohydrates among WF and

MPF. On the other hand, the composition of the two types of breads presented similar values. According to USDA (2019) criteria, commercial white bread should contain approximately the following macronutrient composition: humidity 36.42%, total carbohydrates 49.42% protein 8.85%, lipids 3.33%, ash ≤ 1%. The bread prepared with *M. oleifera* showed better parameters regarding the macronutrient composition standards, compared to the WB. Starch available in breads was also analyzed and the statistical analysis reflected that there were no significant differences (Table 1). Åkerberg et al. (1998), have reported that the starch available in different types of bakery products ranges from values of 80 to 100%.

PPC and antioxidant activity

Quantification of polyphenolic and flavonoid compounds was performed in flour and breads (Table 2). The content of the polyphenolic compounds in MPF flour was four times than WF. Saini et al. (2016) documented an abundance of polyphenolic compounds in all parts of *M. oleifera*, except for the roots and seeds. Furthermore, immature pods contain glucosinolates, carotenoids (mainly lutein), monounsaturated fatty acids, vitamin C, and phytosterols (β-sitosterol). On the other hand, the antioxidant capacity of MPF and BMPF was significantly higher than their counterparts (Table 2). Mainly, the antioxidant capacity of vegetables has been attributed to their phenolic compound content. It has been reported that chlorogenic acid, rutin, quercetin, kaemferol, myrcetin, and moringinine are predominantly present in the plant's immature pods (Kuete, 2017). However, *M. oleifera* pods contain other compounds that can neutralize free radicals or transfer electrons, such as isothiocyanates, vitamins, carotenoids, and unsaturated fatty acids, among others (Saini et al., 2016). This contribution of phytochemicals to the WB is considerable and potentiates the nutraceutical value of this food.

PPC released with dietary fiber

In this study, the BMPF presented almost three times the total dietary fiber content compared to WB. Additionally, BMPF was characterized by having a greater amount of insoluble dietary fiber than its counterpart. The USDA (2018) establishes a total dietary fiber content of 2.70% for white breads, a value entirely below 5.41% found in BMPF. The consumption of food with a high content of dietary fiber is recommended because of its health benefits. It has been reported that dietary fiber has beneficial physiological functions related to blood pressure, serum cholesterol levels, improves blood glucose and insulin sensitivity in non-diabetic and diabetic individuals, and helps in losing weight in obese individuals, as well. Moreover, an increased dietary fiber intake improves several gastro-intestinal disorders, including gastroesophageal reflux disease, duodenal ulcer, diverticulitis, constipation, and hemorrhoids (Anderson et al., 2009).

Table 1: Composition of macro-components of flours and breads

Macro-component	Flours		Bread	
	WF	MPF	WB	BMPF
Moisture	12.13±0.86^{a}	8.69±0.09^{b}	44.46±0.96^{a}	44.93±0.27^{a}
Lipids	3.28±0.74^{a}	4.07±0.11^{a}	2.84±0.90^{a}	3.44±0.68^{a}
Protein	10.79±0.55^{b}	17.65±0.10^{a}	9.20±0.60^{a}	10.26±0.12^{a}
Ash	0.53±0.42^{b}	8.11±0.05^{a}	1.05±0.21^{b}	1.95±0.17^{a}
Carbohydrates*	70.36	44.57	42.45	39.42
Soluble fiber	1.59±0.14^{b}	3.56±0.87^{a}	1.87±0.65^{a}	1.59±0.18^{a}
Insoluble fiber	1.29±1.30^{b}	13.11±2.1^{a}	0.73±0.18^{b}	5.41±0.78^{a}
Starch available	NE†	NE	85.32±1.72^{a}	86.64±0.56^{a}

Values are means±standard deviation of three replicates by triplicate. Means in a column with different superscripts are significantly different under the test of Tukey (p<0.05). *Calculated by difference. †Not estimated.

Table 2: Total soluble polyphenols content, total flavonoids content, antioxidant capacity and bioaccessibility of polyphenols of flours and breads

	Flours		Breads	
	WF	MPF	WB	BMPF
Total phenolics (mg GAE/g)	3.01±1.5^{b}	13.57±1.50^{a}	0.74±0.01^{b}	0.01±0.00^{b}
Total flavonoids (mg CE/g)	0.41±0.12^{a}	1.42±0.30^{a}	3.32±0.02^{a}	0.81±0.17^{a}
ABTS (mmol TE/g)	3.35±0.15^{b}	57.84±4.20^{a}	1.51±0.10^{b}	14.02±0.50^{a}
FRAP (mmol TE/g)	0.77±0.15^{b}	65.6±5.05^{a}	1.18±0.07^{b}	14.69±0.31^{a}
Bioaccessibility of PPC (%)	NE†	NE	77.09	72.99

Values are means±standard deviation of three replicates by triplicate. Means in a column with different superscripts are significantly different under Tukey test (p<0.05). †Not estimated.

Bioaccessibility and kinetics of release of PPC

An *in vitro* enzymatic digestion was performed to simulate the gastric and intestinal stages, which is very useful to understand the release kinetics of PPCs. It was observed that the modified breads presented a higher release of PPC (Fig. 1). A constant release pattern can also be observed in the two samples. A release rate of polyphenolic compounds of 0.078 and 0.058 mg GAE/g was obtained for BMPF and WB, respectively. The release of PPCs depends on the total content of polyphenols and the type of food matrix. In this case we are talking about processed food, so the results contrast with studies where the release of these same compounds in fruits was analyzed (Bouayed et al. 2011; Blancas-Benitez et al. 2015).

Polymeric tannins and hydrolyzable polyphenols are the main polyphenolic compounds associated with dietary fiber and are found mainly in the soluble fraction; they are also the most significant contributors to the antioxidant capacity in dietary fiber (Saura-Calixto, 2011). The polyphenolic compounds associated with the soluble fraction showed a significant difference between WB and BMPF, being higher in the latter. The same behavior was observed for the polyphenols associated with the insoluble fraction. The antioxidant capacity showed a similar trend to that of the polyphenol content. In both food matrices, dietary fiber was shown to function as a transport agent for polyphenolic compounds, with the ability to withstand the action of digestive enzymes. This property can protect some food until it reaches the colon and then exerts its beneficial properties (Arranz et al., 2010).

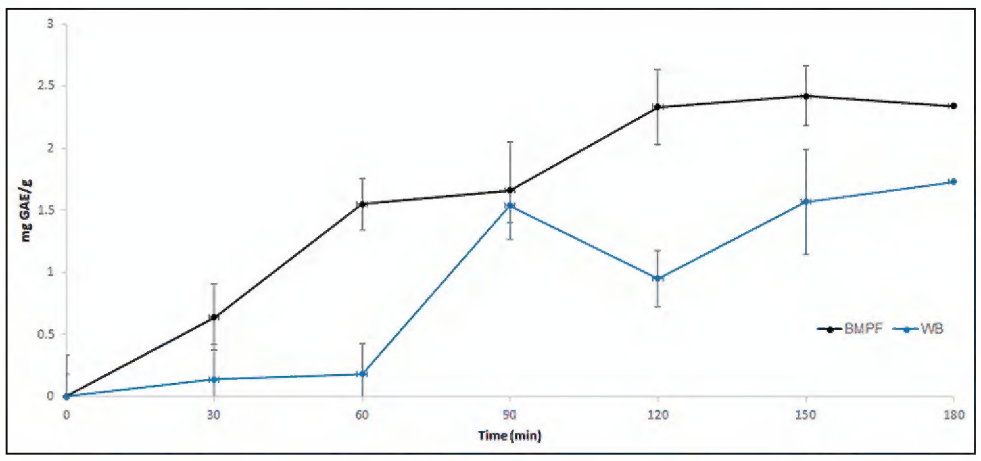

Fig 1. Release behavior of PPC using an *in vitro* digestion model. Data represent the means ± standard error of three replicates.

Glycemic index prediction (pIG)

Fig. 2 shows the degree of starch hydrolysis of the breads analyzed by the enzymatic method. We observed that BMPF presented a lower degree of hydrolysis, followed by WB and the control sample, respectively.

In BMPF, the PPCs in exhibited a decrease in starch hydrolysis. Some studies have shown that extracts of PPCs and purified PPCs can reduce the starch *in vitro* digestion, depending on phenolic composition and molecular structure (Sun and Miao, 2019). According to Granfeldt et al. (1992), dietary fiber and other associated non-fibrous compounds can reduce the rate of starch hydrolysis *in vitro* or *in vivo*, resulting in low metabolic responses. Furthermore, interactions between dietary fiber and added PPCs could result in a tight and compact structure of the starch granule in the crumb. Therefore, this starch entrapment reduces the accessibility to the enzymatic attack and, in consequence, reduces the release of free sugars (Ho et al., 2015). It has also been observed that the PPCs of some plant matrices inhibit the catalytic activity of digestive enzymes such as α-amylase and pancreatic lipase (Martinez-Gonzalez et al., 2017).

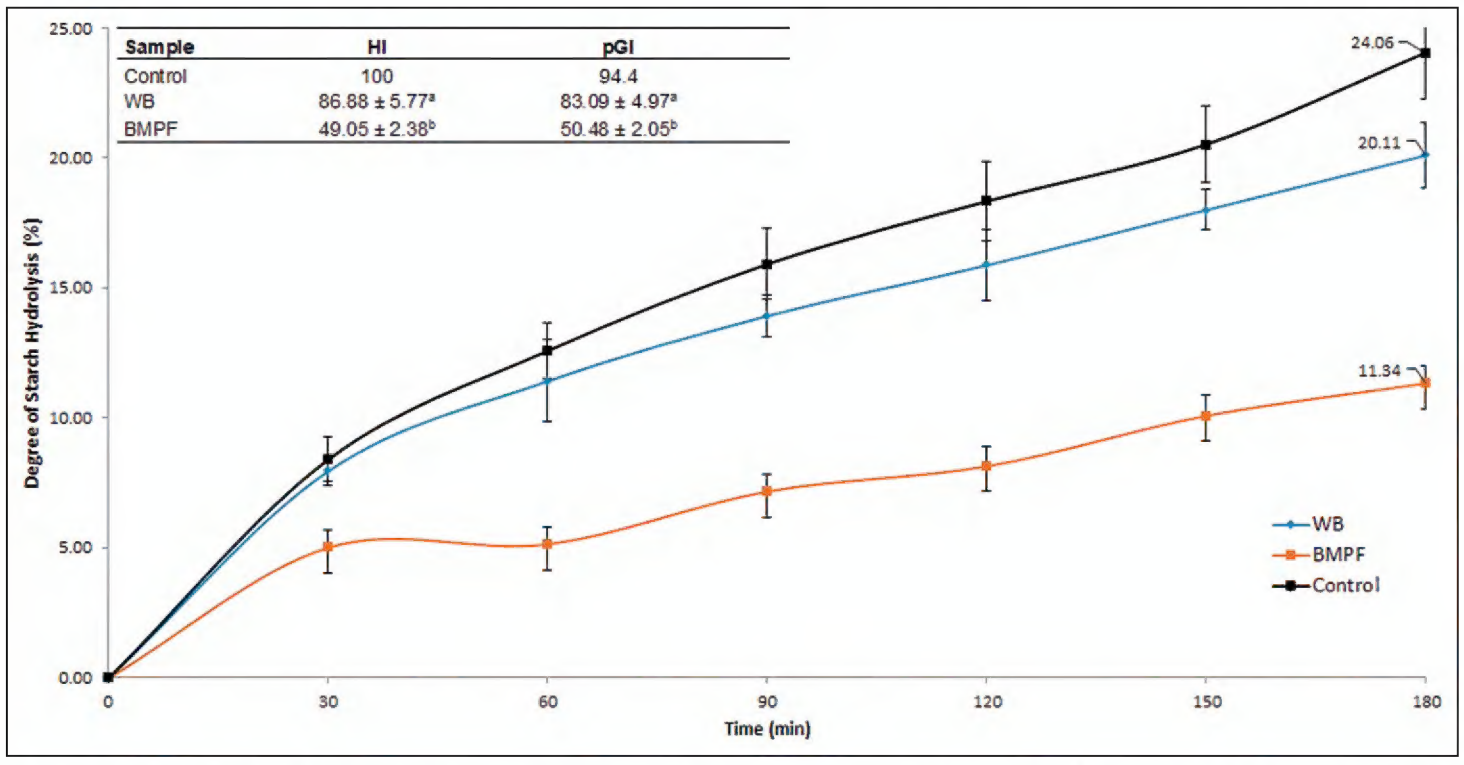

Fig 2. *In vitro* HI of starch and pGI (%). Data are means of six replicates ± standard deviation. Different superscript letters represent significant differences between the types of breads ($p < 0.05$).

Studies with *M. oleifera* leaves have shown a promising glucose-lowering effect in chemically-induced hyperglycemic rats (Azad et al., 2017). Therefore, *M. oleifera* pods could be considered to control hyperglycemic states to control diabetes mellitus type 2. However, the mechanism of action of *M. oleifera* at the tissue level has not yet been revealed.

CONCLUSIONS

The BMPF breads exhibited better results when they were subjected to sensory evaluation. The content of ash, total carbohydrates, and DF presented significant differences among the types of breads, where the DF difference was mainly due to the contribution of IDF by MPF. The substituted bread exhibited a more significant amount of polyphenolic compounds, flavonoids, and antioxidant capacity. The greater amounts of NEPPs are associated with soluble fiber in both types of bread. In general, the bioavailability is similar between the samples and the release kinetics of polyphenolic compounds showed a higher rate in BMPF. These results confirmed our hypothesis since the bread prepared with *M. oleifera* flour increased their antioxidant compounds and fiber. *M. oleifera* is an unconventional source of nutrients and can contribute to improving the composition of traditional foods while aiding to overcome malnutrition, obesity, and metabolic syndrome health problems.

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Conflict of interest

There are none to declare.

Authors' contribution

J.A. Núñez-Gastélum: Conceptualization, supervision, investigation, writing – review and editing; F.E. Maciel-Ortiz: Investigation, general methodology; S.G. Sáyago-Ayerdi: Methodology of kinetics of release of PPC; N.R. Martínez-Ruiz: Methodology of sensory evaluation and analysis of macro-components; E. Alvarez-Parrilla: Formal analysis, review and editing; L.A. de la Rosa: Bioaccessibility of phenolic compounds methodology; J. Rodrigo-García: PPC and antioxidant activity methodology; J.R. Rodríguez-Núñez: Formulation of breads and statistical analysis; G. Mercado-Mercado: Methodology for predicting glycemic index.

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